



Characterization of monoclonal antibodies against feline coronavirus accessory protein 7b



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ABSTRACT

Feline coronaviruses (FCoVs) encode five accessory proteins termed 3a, 3b, 3c, 7a and 7b of unknown function. These proteins are dispensable for viral replication *in vitro* but are supposed to play a role in virulence. In the current study, we produced and characterized 7b-specific monoclonal antibodies (mAbs). A recombinant form of the 7b protein was expressed as a fusion protein in *Escherichia coli*, purified by immobilized metal affinity chromatography and used as immunogen. Two hybridoma lines, 5B6 and 14D8, were isolated that expressed mAbs that recognized 7b proteins of both FCoV serotypes. Using an extensive set of N- and C-terminally truncated 7b proteins expressed in *E. coli* and a synthetic peptide, the binding sites of mAbs 5B6 and 14D8 were mapped to an 18-residue region that comprises the only potential N-glycosylation site of the FCoV 7b protein. The two mAbs were suitable to detect a 24-kDa protein, which represents the nonglycosylated form of 7b in FCoV-infected cells. We speculate that glycosylation of 7b is part of the viral evasion strategy to prevent an immune response against this antigenic site.

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1. Introduction

Feline coronaviruses (FCoVs) are enveloped viruses with a large (approximately 30 kb) single-stranded RNA genome of positive polarity. They belong to the family *Coronaviridae* within the order *Nidovirales* (de Groot et al., 2012). Based on phylogenetic analyses, coronaviruses are divided into four genera termed *Alpha*-, *Beta*-, *Gamma*- and *Deltacoronavirus*. Feline coronaviruses together with the closely related canine coronavirus (CCoV) and porcine transmissible gastroenteritis virus (TGEV) have been classified to the species *Alphacoronavirus 1* in the genus *Alphacoronavirus*. The latter contains a large number of more distantly related alphacoronaviruses, including a number of important medical and veterinary pathogens, such as porcine epidemic diarrhea virus (PEDV), human coronavirus 229E (HCoV-229E), and human coronavirus NL63 (HCoV-NL63) (de Groot et al., 2012). The 5'-proximal two-thirds of the coronavirus genome comprise ORF 1a

and ORF 1b that together encode up to 16 nonstructural proteins (nsps) that form the viral replication/transcription complex but may also be involved in interactions with host cell factors and functions. The remaining part of the genome codes for the structural proteins S (spike), E (envelope), M (membrane) and N (nucleocapsid) (Masters, 2006) and a varying number of so-called accessory proteins with poorly characterized functions.

FCoVs infect cats and other members of the *Felidae* worldwide. Based on serological properties, FCoVs are separated into serotypes I and II (Heeney et al., 1990; Kennedy et al., 2003). Serotype I viruses dominate in the field and account for up to 95% of FCoV infections (Hohdatsu et al., 1992; Addie et al., 2003), whereas the prevalence of type II FCoVs is much lower. Serotype II FCoVs emerged through homologous recombination between serotype I FCoVs and CCoVs (Herrewegh et al., 1998; Terada et al., 2014).

In addition to a serology-based classification, FCoVs may be divided into different biotypes based on their pathogenic potential. The avirulent biotype is generally referred to as feline enteric coronavirus (FECV) and causes inapparent persistent infections of the gut, while the virulent biotype, feline infectious peritonitis virus (FIPV), causes a fatal disease called feline infectious peritonitis (FIP) (Pedersen, 2009). According to the "internal

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mutation theory" FIPV evolves from FECV through mutations in approximately 5–10% of the persistently infected cats (Vennema et al., 1998). So far, the genetic changes responsible for the biotype switch have not been identified, but there is increasing evidence that mutations in the accessory genes and the S gene are involved in the development of FIP (Vennema et al., 1998; Kennedy et al., 2001; Pedersen, 2009; Chang et al., 2010, 2011; Licitra et al., 2013; Bank-Wolf et al., 2014).

Coronavirus genomes comprise a variable number of accessory genes at different positions in the 3'-proximal genome region. With only very few exceptions, homologs of specific accessory genes are only conserved in very closely related viruses (of the same species or sublineage) but not in the more distantly viruses (Lai and Cavanagh, 1997). There is increasing evidence that accessory gene products are important for virulence in the natural host but the precise functions of the vast majority of accessory proteins remain to be investigated. Alphacoronaviruses harbor accessory genes at two different positions in their genomes. Between the S and E genes, FCoV and the very closely related CCoV possess three ORFs (3a–3c), while TGEV contains only two ORFs (3a and 3b) in this genome region. Recently, an additional ORF, named ORF3, was found between the S and E genes in CCoV serotype I (Lorusso et al., 2008). Other alphacoronaviruses, such as PEDV, HCoV-229E and HCoV-NL63, have only one ORF 3. Sequence analyses suggest that FCoV ORF 3a is homologous to CCoV ORF 3a and TGEV ORF 3a while the FCoV ORF 3c is a homolog of CCoV ORF 3c, TGEV ORF 3b and ORF 3 of all other alphacoronaviruses. Downstream of the N gene, all members of the species *Alphacoronavirus 1* contain a different number of additional accessory genes. Thus, TGEV contains only one ORF (called ORF 7), which is homologous to ORF 7a of FCOVs and CCOVs, while the latter two coronaviruses contain yet another ORF called 7b in the 3'-terminal genome region.

Altogether, the FCoV genome encodes five accessory proteins termed 3a, 3b, 3c, 7a and 7b (Dye and Siddell, 2005; Tekes et al., 2008). Using a reverse genetics approach, Haijema et al. (2004) showed that the accessory genes of the FIPV strain 79-1146 are dispensable for viral growth *in vitro* and recombinant viruses that lack ORFs 3a–3c or 7a and 7b were unable to induce FIP *in vivo* (2004). So far, there is no evidence that 3a–3c accessory proteins are produced in infected cells. Nevertheless, it has been proposed that 3c is essential for viral replication in the gut (as is the case for FECV) but dispensable for systemic infections (Chang et al., 2010). The functions of the FCoV-accessory proteins 3a–3c remain to be determined. Recently, it was suggested that the accessory protein 7a represents an interferon antagonist (Dedeurwaerder et al., 2014), although its expression in infected cells has not been confirmed. Among the FCoV-accessory proteins, the 7b protein has been studied most extensively. The 7b protein has a molecular mass of ~26 kDa, it is secreted from the cell and contains (i) a C-terminal KDEL-like endoplasmic reticulum (ER) retention signal, (ii) an N-terminal signal sequence of 17 amino acids and (iii) a potential N-glycosylation site at aa position 68 (Vennema et al., 1992a). The presence of 7b-specific antibodies in both FECV- and FIPV-infected cats indicates that this protein is produced during natural infections (Vennema et al., 1992a,b; Herrewegh et al., 1995; Kennedy et al., 2008). It was also reported that cell culture adaptation often leads to mutations in the 7b gene and loss of expression of this protein (Herrewegh et al., 1995). Taken together, there is growing evidence that the accessory genes and their products are involved in FCoV persistence and virulence but, up to now, their function is not known. One reason for our limited knowledge on FCoV-accessory proteins is the lack of appropriate tools to study these proteins. To our knowledge, specific antibodies against the individual accessory proteins are not available.

In this manuscript, we describe the production of FCoV 7b protein-specific mAbs. Our data show that the mAbs recognize the 7b protein of both serotype I and II FCOVs. Moreover, using a panel of N- and C-terminally truncated 7b proteins and a synthetic peptide, we determined the binding sites of the mAbs in 7b. The data further indicate that the mAbs recognize in FCoV-infected cells only the nonglycosylated form of 7b.

2. Materials and methods

2.1. Ethics statement

All animal procedures were performed in strict accordance with the legal regulations of the German Animal Welfare jurisdiction (Tierschutzgesetz). The animal experiment was approved by the Giessen regional council (Regierungspräsidium) and recorded after approval under reference number A15/2013.

2.2. Viruses and cells

Crandell Rees feline kidney cells (CRFK) were purchased from the American Type Culture Collection (ATCC CCL-94). *Felis catus* whole fetus 4 cells (Fcwf-4) were kindly provided by R. J. de Groot, University of Utrecht, The Netherlands. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 IU ml⁻¹) and streptomycin (100 IU ml⁻¹) in 5% CO₂ at 37 °C. The type-II strain FCoV 79-1146 was kindly provided by R. J. de Groot, University of Utrecht, The Netherlands (GenBank NC_002306). The recombinant type-I FCoV strain recFCoV-ΔStop-7b was generated by reverse genetics as described previously (Tekes et al., 2012). It comprises the type-I FCoV strain Black with a restored accessory gene 7b (GenBank EU186072). The type-II FCoV 79-1146 and the type-I recFCoV-ΔStop-7b were propagated on CRFK cells and Fcwf-4 cells, respectively.

2.3. FIPV infection

CRFK or Fcwf cells were incubated for 1 h with FCoV 79-1146 (multiplicity of infection [MOI] of 1) or recFCoV-ΔStop-7b (MOI of 0.001), respectively. To inhibit N-glycosylation of viral proteins, 2 μg ml⁻¹ tunicamycin in dimethyl sulfoxide (DMSO) were added 4 h post infection (p.i.). At 24 h p.i., the cells were harvested in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% Na-deoxycholate, 0.5 mM Pefabloc SC [Merck], pH 8.0) with 0.2% SDS for SDS-PAGE and Western blot analysis. Following treatment with tunicamycin, cells were either fixed for immunofluorescence or harvested in RIPA buffer for SDS-PAGE and Western blot analysis at 15 h p.i. Deglycosylation of cell lysates was carried out for 3 h with N-glycosidase F (PNGase F; New England Biolabs, MA) according to the manufacturer's instructions.

2.4. Generation of bacterial expression plasmids

The full-length 7b genes of FCoV 79-1146 (nucleotides (nts) 28462–29082) and recFCoV-ΔStop-7b (nts 28337–28954) were amplified by reverse transcription polymerase chain reaction (RT-PCR). The amplified product of FCoV 79-1146 was cloned with primers C32/C33 into the *NcoI/XhoI* sites of a modified pET-11a vector (Novagen). The resulting plasmid was named pC18.2 and contained a C-terminal 12× histidine-tag (7b-His). The amplified product of recFCoV-ΔStop-7b was cloned with primers C83/C82 into a modified pGEX-6P-1 vector (GE Healthcare) by digestion with *BamHI* and *XhoI*. The resulting plasmid with an N-terminal GST and a C-terminal 13× His-tag was named pC80.1 (GST-7bΔStop-His). For the generation of GST-7bΔSS-His (pC52.1; C50/

C47), a deletion mutant of 7b (amino acid 17–206, without the N-terminal signal sequence [Δ SS]) was cloned into the same modified pGEX-6P-1 vector. To determine the antigenic site of the monoclonal antibodies, N- and C-terminally truncated 7b proteins of FCoV 79-1146 were generated as GST-fusion proteins. To this end, the sequences were amplified by PCR with specific primers and cloned into the modified pGEX-6P-1 vector. The resulting plasmids were named pC71.2 (aa 112–206; C72/C47), pC72.1 (aa 1–111; C46/C73), pC82.1 (aa 65–206; C84/C47), pC88.1 (aa 26–206; C86/C47), pC89.2 (aa 34–206; C87/C47), pC90.1 (aa 42–206; C88/C47), pC91.5 (aa 50–206; C89/C47), pC92.9 (aa 58–206; C90/C47), pC97.4 (aa 1–60; C46/C101), pC98.1 (aa 1–63; C46/C102), pC102.3 (aa 1–67; C46/C103), pC108.2 (aa1–70; C46/C107) and pC114.1 (aa 1–75; C46/C109). Primer sequences are shown in Table 1.

The pET-11a vector was modified by PCR with primers C17 and C18 to insert an *NcoI* and *XhoI* restriction site upstream of a *BamHI* restriction site. Furthermore, a 7 $\times$ His-tag followed by a stop codon behind the *XhoI* site was inserted and the existing *BamHI* site was deleted. In a second PCR with primers C12 and C18, the His-tag was extended with 5 histidines to generate a 12 $\times$ His-tag. The pGEX-6P-1 vector was modified by PCR with primers C36 and C37 to insert a 13 $\times$ His-tag downstream of the existing *XhoI* site. Plasmid constructs were verified by sequence analysis.

2.5. Protein expression and affinity purification

The expression of the recombinant proteins was performed after transformation of the different plasmids into *Escherichia coli* (*E. coli*) strain RosettaTM(DE3) pLysS (Novagen). An overnight culture was diluted 1:10 in fresh LB medium supplemented with ampicillin (100 μ g ml⁻¹), chloramphenicol (34 μ g ml⁻¹) and glucose (1%) to a final volume of 500 ml and grown for 2–3 h at 37 °C to OD₆₀₀ of 1.0. Protein expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 3 h of growth at 28 °C the cells were harvested by centrifugation and resuspended in 50 mM Na₂HPO₄, 300 mM NaCl and 1% Triton X-100. Cells were lysed by freeze/thaw treatment (3 $\times$) and sonication on ice. The insoluble protein

fraction was separated from soluble proteins by ultracentrifugation (100,000 $\times$ g for 1 h). To solubilize inclusion bodies the pellet was resuspended in 8 M urea, 300 mM NaCl and 50 mM Na₂HPO₄, pH 8.0 or in 6 M guanidinium-hydrochloride (GuHCl), 50 mM Na₂HPO₄, 20% ethanol, 1% Triton X-100 and 100 mM NaCl, pH 8.0 and treated with ultrasonication. After an additional ultracentrifugation at 100,000 $\times$ g for 30 min, the solubilized proteins were applied on HisTrap HP columns (GE Healthcare). The purification of the recombinant proteins was performed by metal ion affinity chromatography (IMAC) with Ni²⁺ Sepharose as recommended in the supplier's protocol. The purified recombinant proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Following electrophoresis the proteins were identified by Western blot using anti-His mAb. After dialysis overnight against PBS (GST-7b- Δ SS-His) or acetone precipitation (7b-His), the proteins were used for the immunization of mice. Protein concentrations were determined in Bradford assay (Bradford, 1976).

2.6. Preparation of monoclonal antibodies (mAbs)

Four 12 week old female BALB/c mice (Charles River Breeding Laboratories) were injected subcutaneously with 50 μ g of purified protein GST-7b Δ SS-His in Freund's incomplete adjuvant (Sigma) on days 0, 22 and 54. Antibodies binding the recombinant protein were detected in serum samples by Western blotting after the third injection. On days 70, 71 and 72 the mouse with the best immune response was immunized with 40 μ g of purified protein 7b-His without adjuvant. One day after the final antigen application splenocytes were fused with sp2/0-Ag14 myeloma cells (ATCC CRL-1581) at a ratio of 3:1 using polyethylene glycol 1.500 (Roche) according to standard protocols (Köhler et al., 1976). The fused cells were cultured in 96-well plates and hybridoma cells were selected in DMEM supplemented with 20% F10 (GIBCO), 20% F12 (GIBCO), 15% FCS, 2 mM L-glutamine, 0.35% 2-mercaptoethanol, 50 mM HEPES, oxaloacetate-pyruvate-insulin supplement (OPI; Sigma), and hypoxanthine-aminopterin-thymidine supplement (HAT; Sigma).

2.7. Indirect ELISA screening

For the detection of specific antibodies against the 7b protein, hybridoma culture supernatants were screened by ELISA (Engvall and Perlmann, 1971) 7–10 days after fusion. To avoid screening for antibodies that react with GST, the fusion protein 7b-His was used for coating. However coating with 7b-His resulted in OD_{450nm} values <0.35. As an alternative flat-bottomed ELISA 96-well plates (Nunc MaxiSorp) were coated overnight at 4 °C with 35 ng purified GST-7b Δ SS-His protein in 0.1 M Na₂CO₃/NaHCO₃ (pH 9.5) per well. The supernatants were also tested against an irrelevant control protein (GST-YFP-His; 75 ng/well) in order to exclude GST- and His-reactive clones. This control protein was expressed in the same vector and purified as described for the GST-7b Δ SS-His construct. After washing three times with PBST (PBS containing 0.1% Tween-20), the plates were blocked with 10% FCS in PBST for 1 h at 37 °C. Then, the plates were washed again and incubated with 100 μ l/well of hybridoma culture supernatants. Specific antibodies were detected using goat anti-mouse IgG conjugated with horseradish peroxidase (Dianova) at dilution 1:2500. The ELISA was performed with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) and enzyme reactions were terminated with 25% (v/v) H₂SO₄ solution. The optical density (OD values) at 450 nm was measured in a microtiter plate reader (Spectra II, SLT). Hybridomas with ELISA values >0.4 were cloned twice. Reactivity was confirmed by ELISA and specificity was determined by Western blot analysis. To confirm the antigenic sites recognized by the mAbs 5B6 and 14D8, ELISA

Table 1
Primer sequences used to amplify defined fragments of FCoV ORF 7b.

Primer	Sequence 5'-3'
C12	acatcatcatcatcatcatcaccaccaccaccac
C17	caccaccaccaccaccaccactaacacgagaagatcc
C18	CTCGAGaaaCCATGGcatatgtatctctcttaaag
C32	aaaCCATGGcatgtgtgttaactcttg
C33	tttCTCGAGtttaactcagttttatgatgttg
C36	caccaccaccaccaccaccaccgcccgcacgtgactgac
C37	ctaagtgtgatgtgatgtgatgCTCGAGtttagtgatgtgtgatg
C46	aaaGGATCCcatgtgtgtgttaactcttg
C47	tttCTCGAGtaactcagttttatgatgttg
C50	aaaGGATCCactgctgtgcaaatgacc
C72	aGGATCCagattggtgtgtgtgtg
C73	tCTCGAGgagattgattcttggattccc
C82	tCTCGAGtaactcagttttacgatgttg
C83	aGGATCCcatgtgtgtgtactctgtgtg
C84	aGGATCCcatggaagtttcaattgcac
C86	aGGATCCcatggaacatcccttcttac
C87	aGGATCCatgtttattacagcatttcatagg
C88	aGGATCCcatgacctctacattacaacac
C89	aGGATCCatggtcttagcactacc
C90	aGGATCCcatgctgtgtgtgtgtg
C101	tCTCGAGctcaacacgagatcc
C102	tCTCGAGaccctcactcaac
C103	tCTCGAGaaaccttcgataccttc
C107	tCTCGAGtgtcaattgaaaccttg
C109	tCTCGAGttgaaagccagccatg

plates were coated with 460 ng/well of BSA-7b-pep (C-RVECE-GIEGFNCTWPGFQ [Centric Biotec, Germany]). Due to problems during the synthesis of the peptide, the internal cysteines were linked by a disulfide bond. Therefore, the peptide was incubated with the reducing agent DTT (5 mM) for 1 h at room temperature before coating. As negative control, an irrelevant peptide (coupled to BSA), BSA-7b-CLVG (1 µg/well; C-LVGAVPKQKRLNVG, BioGenes, Germany) and, as positive control, the purified protein GST-7bΔSS-His (36 ng/well) were coated. The mAbs 5B6 and 14D8 were used at a 1:3 dilution, anti-His mAb was used at a 1:10 dilution. The ELISA was performed as described above.

2.8. Determination of immunoglobulin isotype

The immunoglobulin subclass of both mAbs was determined using the commercially available Iso-Gold Rapid Mouse-Monoclonal Isotyping Kit (BioAssay Works) according to the manufacturer's instructions.

2.9. SDS-PAGE and Western blotting

SDS-PAGE was carried out using a 10% tricine-polyacrylamide gel system (Schagger and von Jagow, 1987). For Western blot analysis, the proteins were transferred to a nitrocellulose membrane (Pall Corporation). Residual binding sites were blocked with 4% skimmed milk in PBST. The membrane was then incubated with monoclonal antibodies of the hybridoma cell culture supernatants (primary antibodies) at a 1:3 dilution or sera in PBST (1:1500 dilution). Bound antibodies were visualized with HRP-conjugated goat anti-mouse or goat anti-cat IgG (Dianova) and chemiluminescence reagent (Western Lightning Plus-ECL; PerkinElmer). Goat anti-cat IgG was diluted in PBST with 1x Roti®-Block (Roth) additionally.

2.10. Indirect immunofluorescence assay

CRFK cells were cultured on sterile cover slips in 24 well plates pretreated with rat tail collagen (Sigma). Cells were mock or virus-infected and treated with 2 µg ml⁻¹ tunicamycin 4 h p.i. At 15 h p.i. the cells were fixed with PBS containing 4% paraformaldehyde for 20 min at 4°C, followed by three PBS washes. Cells were permeabilized with Triton X-100 (1% in PBS) for 5 min at room temperature. The cells were rinsed twice with PBS and residual binding sites were blocked with 1× Roti®-ImmunoBlock (Roth) for 10 min at room temperature. The cells were then incubated with primary antibodies (1:3 dilution) or sera (1:1500 dilution) in PBS for 1 h at 37°C, followed by three PBS washes. Subsequently the cells were incubated with secondary antibodies conjugated to cyanogen-3 (1:500 dilution, Dianova) in PBS for an additional hour at 37°C. The DNA was counterstained with 66 µg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. After three washes the cover slips were mounted with Mowiol (Sigma) containing the anti-fading reagent DABCO (1,4-diazabicyclo(2.2.2)octane, Roth). The immunofluorescent staining was visualized by fluorescence microscopy (Axiovert 10, Zeiss).

3. Results

3.1. Expression and purification of FCoV 7b fusion protein

The full-length 7b protein of FCoV 79-1146 of 206 amino acid (aa) residues was expressed in *E. coli* with a C-terminal His-tag (7b-His). Induction of expression of 7b-His resulted in insoluble protein aggregates that could be solubilized using 6 M GuHCl-containing buffer and purified by ion metal affinity chromatography (IMAC). The identity of the purified protein was confirmed by SDS-PAGE and Western blotting using anti-His mAb. The protein had an apparent molecular mass of 24 kDa as judged by SDS-PAGE analysis, which is predicted for this protein, and was recognized

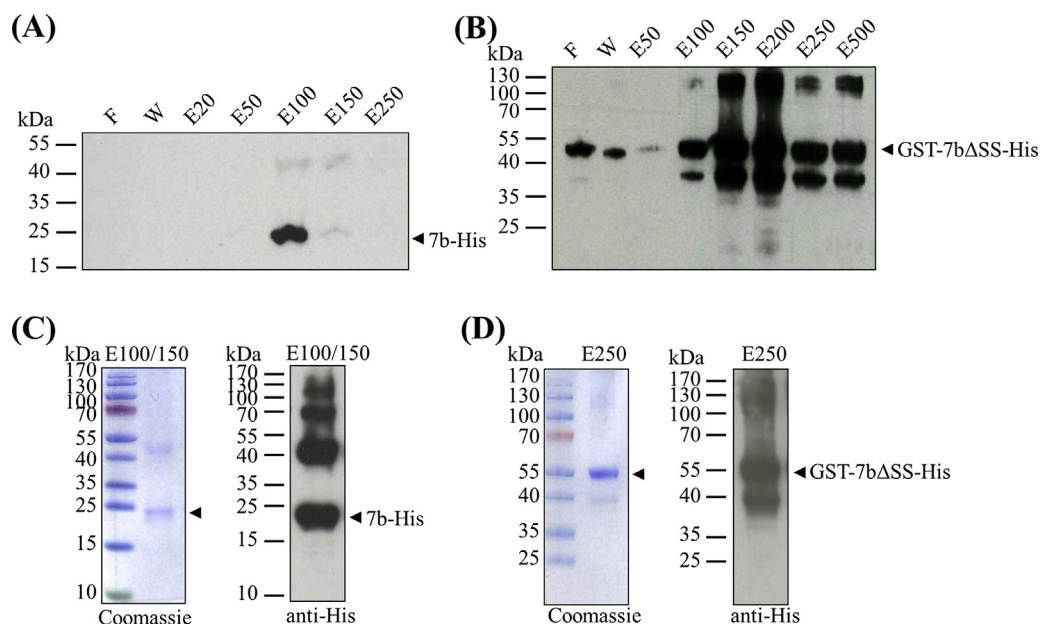


Fig. 1. Purification of recombinant proteins 7b-His and GST-7bΔSS-His. Recombinant proteins 7b-His and GST-7bΔSS-His were purified from *E. coli* by Ni²⁺ affinity chromatography using buffers containing 6 M GuHCl and 8 M urea, respectively, and analyzed by SDS-PAGE under non-reducing conditions. (A and B) Western blot analysis (anti-His mAb) of protein samples obtained at different steps of the purification of 7b-His (A) and GST-7bΔSS-His (B). (E and D) Eluate fractions containing 7b-His (E) and GST-7bΔSS-His (D) were pooled, concentrated and analyzed by SDS-PAGE under non-reducing conditions followed by Coomassie staining (left) or Western blotting using a His-tag-specific antibody (right). Arrowheads indicate positions of the purified recombinant proteins. F: flow-through fraction; W: wash fraction; E: eluate fractions obtained with different concentrations of imidazole (numbers indicate concentrations [mM]).

by the His-tag-specific antibody (Fig. 1a). Western blot analysis further revealed a protein of approximately 48 kDa, suggesting dimerization of the full-length protein. The overall yield for this protein was low. We therefore decided to express 7b as part of a GST fusion protein. To do this, the 5' region encoding a putative N-terminal signal sequence (SS, residues 1 to 17) was removed from the 7b coding sequence and replaced by the GST coding sequence. The resulting construct was termed GST-7b Δ SS-His. Expression of GST-7b Δ SS-His in *E. coli* resulted again in insoluble protein aggregates. The protein was purified under denaturing conditions using 8 M urea-containing buffer and analyzed by SDS-PAGE and Western blotting using an anti-His mAb. Consistent with its calculated molecular mass, the major fraction of the purified protein migrated as a 51-kDa protein in SDS polyacrylamide gels. Two additional minor bands in the SDS-PAGE were specifically recognized by Western blotting using the His-tag-specific mAb. These bands are consistent with a dimer and a 37-kDa degradation product, respectively, of the GST-7b Δ SS-His protein (Fig. 1b). The total yield of GST-7b Δ SS-His was estimated to be 25 times higher compared to 7b-His. The elution fractions of 7b-His and GST-7b Δ SS-His were pooled, concentrated, desalted and used for immunization (Fig. 1c and d, respectively).

3.2. Immunization of mice with purified recombinant FCoV 7b fusion protein and identification of positive hybridoma cell lines

The fusion protein 7b-His was used for the immunization of BALB/c mice. However, even after four injections over a time period of 12 weeks, no immune reaction against 7b-His could be detected in sera obtained from immunized animals. We therefore decided to immunize another four BALB/c mice using the GST-7b Δ SS-His protein. Two weeks after the third injection a specific seroconversion was detected. The mouse with the best immune response received an additional boost with 7b-His. Splenocytes isolated from immunized mice were fused with myeloma cells and cell culture supernatants were screened by ELISA using the GST-7b Δ SS-His as capture antigen. GST-YFP-His was used as a control antigen to identify and exclude clones producing antibodies specific for one of the tags. Two supernatants were confirmed to contain antibodies that bind to GST-7b Δ SS-His but not the GST-YFP-His control protein. The respective hybridoma cells were subcloned twice and continued to produce 7b-specific antibodies as determined by ELISA. Antibodies produced by the two stable hybridoma cell lines (5B6 [IgG2a], 14D8 [IgG1]) were subsequently tested by Western blotting. Both mAbs recognized the recombinant proteins 7b-His (Fig. 2a) and GST-7b Δ SS-His (data not shown). The data also suggest that the formation of the putative 7b dimer discussed above (Fig. 1c and text) involves one or more intermolecular disulfide bonds since the higher molecular mass band of >40 kDa was not detectable if 2-mercaptoethanol was included in the protein sample buffer (Fig. 2b).

3.3. Reactivities of anti-7b mAbs with serotype I FCoV 7b protein

To determine whether the two FCoV serotype II (strain 79-1146) 7b protein-specific mAbs recognize epitopes that are conserved among serotype I and II strains, the mAb reactivities against the well-characterized serotype I strain Black were analyzed. The 7b proteins of strains Black and 79-1146 share 90% amino acid sequence identity. However, strain Black contains an internal translation stop codon in ORF 7b, resulting in a C-terminally truncated 7b protein. We therefore restored the ORF 7b of FCoV strain Black and expressed the strain Black 7b peptide sequence as part of a recombinant fusion protein (GST-7b Δ Stop-His) in *E. coli*. Western blot analysis of lysates obtained from IPTG-induced *E. coli* cells transformed with the appropriate expression plasmid

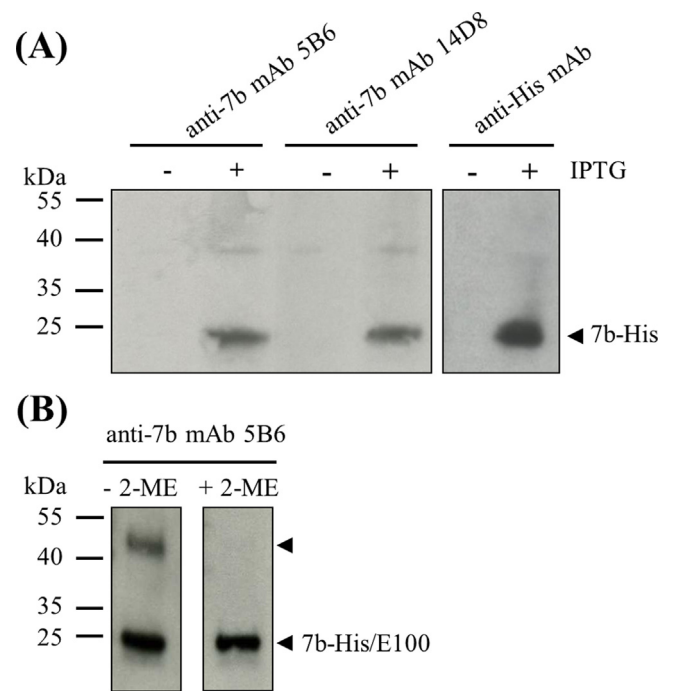


Fig. 2. Specific binding of mAbs to a C-terminally His-tagged form of the FCoV 79-1146 7b protein (7b-His) expressed in *E. coli*. (A) Specificities of 5B6 and 14D8 were characterized using total lysates of noninduced (–) and IPTG-induced (+) *E. coli* Rosetta cells transformed with the appropriate 7b-His expression plasmid (see Materials and Methods). Proteins were separated by SDS-PAGE (10%) under non-reducing conditions and antibody reactivities were analyzed by Western blotting using anti-7b mAbs (5B6 and 14D8, respectively) and anti-His mAb (control) as indicated. (B) The purified protein 7b-His/E100 was separated by SDS-PAGE (10%) in the absence (–2-ME) or presence (+2-ME) of the reducing agent 2-mercaptoethanol. Western blot analysis was performed using anti-7b mAb 5B6. Arrowheads indicate the recombinant protein 7b-His. E100: Eluate fraction obtained with 100 mM imidazole.

confirmed that the two mAbs recognized the recombinant 7b protein of strain Black (data not shown), confirming that the two mAbs obtained in this study recognize epitopes that are conserved among the 7b proteins of representative FCoV serotype I and II strains.

3.4. Identification of mAb binding sites in the FCoV 7b protein

To further characterize the newly generated mAbs, we sought to determine the binding sites within the 7b protein. As shown above, both mAbs recognized 7b-His in Western blot experiments after SDS-PAGE under non-reducing and reducing conditions, suggesting that they recognize a linear epitope on the 7b protein. First, we produced a set of bacterially expressed segments of 7b. Appropriate coding sequences were cloned into the pC23.6 plasmid and expressed with N-terminal GST and C-terminal histidine tags. mAb reactivities against these proteins were tested by Western blotting (Fig. 3). All proteins containing aa residues 58–111 of 7b (pC72.1, pC88.1, pC89.2, pC90.1, pC91.5, pC92.9) were detected by the mAbs. In contrast, proteins containing residues 112–206 (pC71.2) or 65 to 206 (pC82.1) were not recognized. Furthermore, a protein containing residues 1–75 of 7b (pC114.1) was recognized while none of the slightly smaller proteins that lacked a few more residues at the carboxyl terminus (pC97.4, pC98.1, pC102.3, pC108.2) were recognized. The combined results summarized in Fig. 3 led us to conclude that both mAbs recognize a peptide segment that includes residues 58 to 75 of the 7b protein (Fig. 3).

To further corroborate this hypothesis, a synthetic peptide coupled to BSA (BSA-7b-pep, C-⁵⁸RVECEGIEGFNCTWPGFQ⁷⁵) was

Plasmid	Amino acid	Detection by western blot	
		5B6 mAb	14D8 mAb
pC18.2 (7b-His)	1 206	+	+
pC72.1	1 111	+	+
pC71.2	112 206	-	-
pC88.1	26 206	+	+
pC89.2	34 206	+	+
pC90.1	42 206	+	+
pC91.5	50 206	+	+
pC92.9	58 206	+	+
pC82.1	65 206	-	-
pC97.4	1 60	-	-
pC98.1	1 63	-	-
pC102.3	1 67	-	-
pC108.2	1 70	-	-
pC114.1	1 75	+	+

Fig. 3. Determination of the 5B6 and 14D6 binding sites in the 7b protein. The scheme shows the full-length 7b construct and the truncated versions derived from this construct. All proteins were expressed as GST- and His-tag fusion proteins and mAb reactivities were tested by Western blotting under non-reducing conditions. +: specific binding detected; -: not detected.

used in an additional set of experiments. To resolve problems that occurred during peptide synthesis, an intramolecular disulfide bond was introduced. To further characterize the mAb binding properties, BSA-7b-pep was used as antigen in an ELISA that was performed under non-reducing and reducing conditions, respectively. BSA-7b-pep was treated with or without DTT and ELISA plates were coated with the peptide. BSA-7b-CLVG and purified GST-7b Δ SS-His fusion protein were used as negative and positive control, respectively. As shown in Fig. 4, mAbs 5B6 and 14D8 were confirmed to bind specifically to BSA-7b-pep in the absence and presence of DTT. The data provide direct evidence that both mAbs recognize a region in the 7b protein that spans residues 58 to 75.

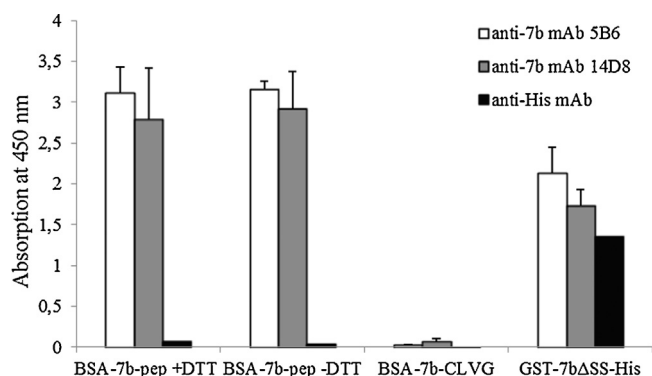


Fig. 4. Reactivities of mAbs 5B6 and 14D8 against BSA-7b-pep as determined by ELISA. ELISA plates were coated with BSA-7b-pep in the presence (+) or absence (–) of DTT. As controls, BSA-7b-CLVG and GST-7b Δ SS-His were used. The data represent three independent experiments; standard deviations are indicated.

3.5. Comparative sequence analysis of the peptide region recognized by the mAbs

As shown above, the epitope(s) recognized by both mAbs are located within the amino acid sequence 58 to 75 of the FCoV 7b protein. This part of the 7b sequence is highly conserved within FCoV serotypes I and II with sequence identities of 78–94%. Interestingly, this part of the sequence comprises the only *N*-glycosylation motif (⁶⁸NCT⁷⁰) within the 7b sequence (Fig. 5). This motif is conserved in almost all sequenced FCoV strains.

3.6. Detection of accessory protein 7b in FCoV-infected cells

In order to detect the authentic 7b protein with our newly generated anti-7b mAbs, CRFK cells were mock-infected or infected with FCoV 79-1146 and analyzed by indirect

		..				
		60		70		75
FCoV Serotype II	79-1146	RVE	CEGIEGF	NCT	WPGFQ	
	UCD6-1	RVE	CESIEGF	NCT	WPGFQ	
	NTU156	RVE	CESVEGF	NCT	WPGFQ	
	79-1683	RVE	CESVDGF	NCT	WPGFQ	
FCoV Serotype I	Black	RVE	CESIEGF	NCT	WPGFQ	
	UCD1	RVE	CESIEGF	NCT	WPGFQ	
	UU18	RVE	CESVEGF	NCT	WPGFQ	

Fig. 5. Multiple sequence alignment of ORF 7b-encoded amino acid sequences from position 58–75 of representative FCoV strains: 79-1146 (AY994055), UCD6-1 (ACR46154), NTU156 (ACS44225), 79-1683 (CAA47250), Black (EU186072), UCD1 (CAA62201) and UU18 (ADL71463). Differences in the putative binding sequence from aa 58–75 of other strains are highlighted by grey boxes. The *N*-glycosylation site (⁶⁸NCT⁷⁰) is depicted by a clear box.

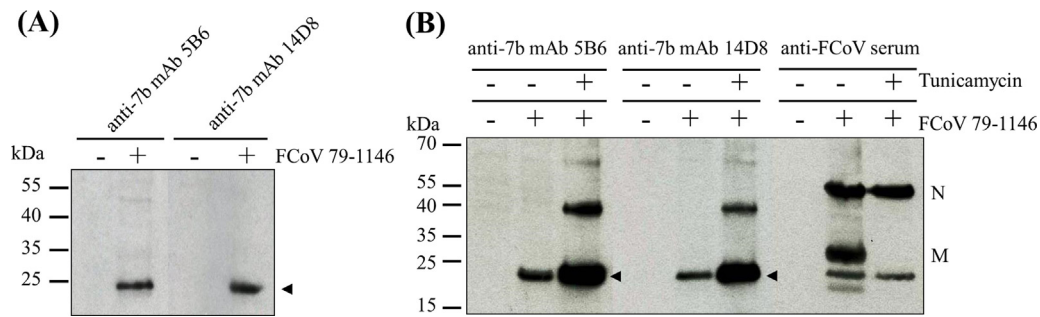


Fig. 6. Detection of FCoV 79-1146 7b protein by Western blot. CRFK cells were infected at an MOI of 1 (+) or mock-infected (–). (A) Cells were harvested at 24 h p.i. and cell lysates separated by SDS-PAGE (10%) under non-reducing conditions. Western Blot was performed with anti-7b mAbs 5B6 and 14D8. (B) At 4 h p.i., the cells were either treated with tunicamycin (+) or left untreated (–). 15 h p.i. cell lysates were subjected to SDS-PAGE (10%) under non-reducing conditions and immunoblotted with anti-7b mAbs or feline anti-FCoV serum. Virus-specific proteins N and M are indicated. The arrowheads indicate the 7b protein band of the monomer. Please note that the assumed dimer of FCoV 7b migrates slightly slower than expected. This could be due to peculiar migration behaviour of the dimer. Alternatively, proteolytic degradation may have occurred.

immunofluorescence assay (IFA) 24 h post infection (p.i.). Surprisingly, neither of the anti-7b mAbs produced a positive signal, although there was a clear reactivity with an antiserum against FCoV (data not shown). As the two mAbs were shown previously to

recognize FCoV 7b fusion proteins in Western blot experiments (Fig. 2), we used this method to analyze cell lysates obtained at 24 h p.i. Both mAbs detected a protein with an apparent molecular mass of approximately 24 kDa in FCoV-infected cells, whereas no signal

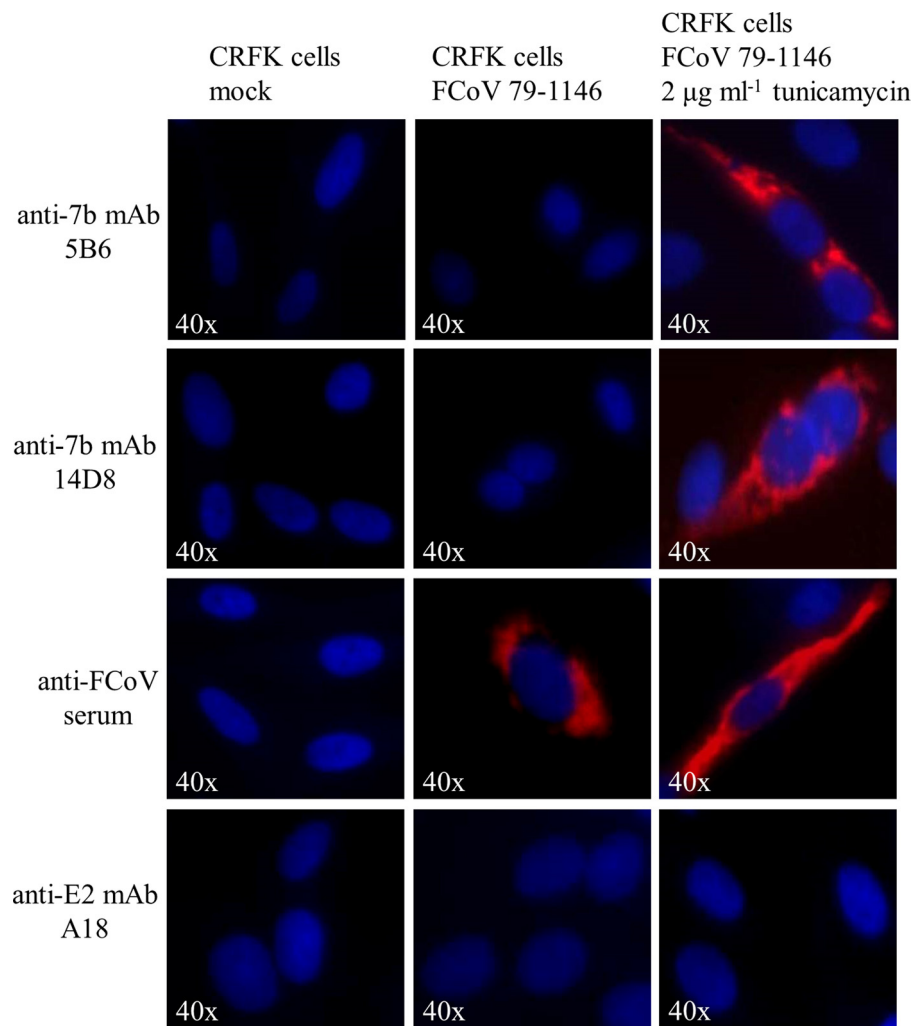


Fig. 7. Detection of 7b protein after tunicamycin treatment by indirect immunofluorescence assay. CRFK cells were mock infected (left panel) or infected with FCoV 79-1146 at an MOI of 1 (middle and right panels). At 4 h p.i., infected cells were treated with tunicamycin (right panel) or left untreated (middle panel) and cells were fixed 15 h p.i. Immunofluorescent staining with the anti-7b mAbs 5B6 and 14D8 revealed the presence of the nonglycosylated 7b protein in the cell cytoplasm. The anti-FCoV serum was used as positive control and anti-E2 mAb A18 (mAb against E2 of a pestivirus) as negative control. Cell nuclei were stained blue with DAPI.

was detected in mock-infected cells (Fig. 6a). It has been shown by others that ORF 7b encodes a glycoprotein of 26 kDa (Vennema et al., 1992a). In order to investigate whether our mAbs detect a glycosylated form of the 7b protein, PNGase F treatment of cell lysates was performed. Surprisingly, incubation with PNGase F did not affect the migration behavior of the 7b protein (data not shown), indicating that the protein detected by the mAbs represents a nonglycosylated form of the 7b protein.

Since the only N-glycosylation motif in 7b is located at aa position 68–70 (NCT), we speculated that the N-glycosylation site might be part of the epitope(s) recognized by the mAbs. Accordingly, the epitope(s) may be masked and thereby inaccessible for our antibodies. In order to test this hypothesis, the effect of an inhibitor of N-linked glycosylation was studied. CRFK cells were infected with FCoV 79-1146 or mock-infected and treated with tunicamycin at 4 h p.i. Cell lysates were prepared at 15 h p.i. and probed by Western blotting. The mAbs recognized a single protein with an apparent molecular mass of 24 kDa in the absence and presence of tunicamycin (Fig. 6b). Interestingly, the signal intensity obtained with the mAbs was much stronger after inhibition of N-glycosylation. Moreover, after treatment with tunicamycin, a dimer of 7b was recognized by both mAbs which was not detectable in the absence of tunicamycin (Fig. 6b) and disappeared after incubation with 2-mercaptoethanol (data not shown). These results support the idea that both mAbs do not recognize the glycosylated form of FCoV 7b. An anti-FCoV serum, which served as positive control, detected the virus-specific proteins N and M (Fig. 6b). In addition, a protein band with an apparent molecular mass of 24 kDa was detected by the anti-FCoV serum. This protein may represent the nonglycosylated form of the M protein and/or the 7b protein.

The results outlined above show that the anti-7b mAbs recognize exclusively the nonglycosylated form of the viral protein in FCoV-infected cells. This was particularly striking after treatment of the cells with tunicamycin. In light of this finding, another round of immunofluorescence experiments was performed with the mAbs after infection with FCoV 79-1146 and incubation with tunicamycin. As already mentioned, there was no staining after incubation with the mAbs in the absence of tunicamycin. However, after tunicamycin treatment, clear signals were obtained in infected cells (Fig. 7). At this point, the observed cytoplasmic localization of 7b cannot be further narrowed down. An anti-FCoV serum detected viral proteins in the cytoplasm of infected cells regardless of whether or not they were treated with tunicamycin (Fig. 7).

4. Discussion

The genome of feline coronavirus (FCoV) encodes five accessory proteins termed 3a–3c, 7a and 7b. Among these, only 7b has been identified in virus-infected cells (Vennema et al., 1992a). The 7b protein was also expressed from a plasmid under the control of the T7 promoter in T7 polymerase-expressing vaccinia virus-infected cells. The produced 7b protein was identified with ascites from a FIPV-infected cat. FCoV 7b was shown to be a glycoprotein that to some extent may be secreted from infected cells. Interestingly, a KDEL-like sequence at its C-terminus has been shown to slow down the export of the protein (Vennema et al., 1992a).

We are interested in accessory proteins encoded by FCoV in particular with respect to potential roles in (i) establishing persistent infections by enteric feline coronavirus in the gut and (ii) the pathogenesis of feline infectious peritonitis (Pedersen, 2009). Since there was already some information on FCoV 7b, we first concentrated on this protein. For further characterization including its unambiguous identification in FCoV-infected cells monoclonal antibodies against 7b protein were generated. Using

N- and C-terminally truncated parts of the 7b protein expressed by bacteria as well as a synthetic peptide conjugated to BSA, the binding sites of two mAbs were determined. According to this analysis, the mAbs recognize a stretch of 18 amino acids (aa) between aa position 58 and 75. Interestingly, the only putative N-linked glycosylation site as well as two of the seven cysteines of the FCoV 7b protein are contained within this sequence. This finding led to the question whether the mAbs recognize only the nonglycosylated form of 7b. In fact, both mAbs detected in FCoV-infected cells only recognized a protein with an apparent molecular weight of 24 kDa and not the previously described glycosylated form of 26 kDa (Vennema et al., 1992a). The exclusive recognition of the nonglycosylated form was supported by results obtained after tunicamycin treatment of FCoV-infected cells. Notably, immunofluorescence studies using the mAbs led to negative results unless the FCoV-infected cells were treated with tunicamycin. The difference to Western blot experiments where the nonglycosylated form could be readily shown without inhibition of glycosylation most likely results from different sensitivities of the two methods. Masking of epitopes by glycosylation has been shown for many other viral proteins including the hemagglutinin of influenza virus (Skehel et al., 1984). The authors also used tunicamycin to demonstrate recognition by mAbs in the absence of N-linked glycosylation. Accordingly, the antigenicity of influenza virus hemagglutinin and of other proteins can be modified by addition of carbohydrate chains. Furthermore, since both mAbs recognize the same or a nearby epitope in 7b, it can be assumed that the identified 18 aa stretch represents a highly antigenic site. Masking of this region through glycosylation of asparagine 68 may prevent an immune response and could be relevant for establishment of persistent infection by FCoV. This could be directly tested by the use of recombinant FCOVs, where the N-linked glycosylation site of 7b is missing. It remains to be seen whether this finding has implications for vaccine development against FCoV-induced diseases.

Another interesting aspect of our studies with FCoV 7b concerns putative intra- and intermolecular disulfide bonds. In this context, it is noteworthy that expression of 7b in bacteria allowed the detection of disulfide linked dimers, which disappeared after treatment of extracts with a reducing agent. Such dimeric forms were also detected in FCoV-infected cells, but apparently only after treatment with tunicamycin. Thus, it is tempting to speculate that intermolecular disulfide bridges represent an artifact only observed in the absence of glycosylation at asparagine 68 of FCoV 7b. It will be interesting to determine whether one or both cysteines present in our synthetic peptide is/are involved in intramolecular disulfide bonds. Recognition of 7b by our mAbs in the absence and presence of reducing agent may give some indirect evidence in this regard. Pilot experiments actually indicate that our mAbs recognize the nonreduced form of 7b from FCoV-infected cells better than the reduced one; this result suggests that the native protein contains one or more intramolecular disulfide bridges, which are important for the antigenicity of 7b. Another way to study the relevance of disulfide bonds for the structure of 7b is the exchange of cysteine codons in different expression systems together with Western blots under nonreducing and reducing conditions. However, final proof for the presence of intramolecular disulfide bridges within FCoV 7b will require the determination of its three-dimensional crystal structure.

Unfortunately, our mAbs are not well suited to detect the secreted glycosylated form of 7b. To further study the synthesis, intracellular localization and secretion of 7b, we will use our reverse genetics system (Tekes et al., 2008, 2012) to obtain recombinant viruses containing the desired changes in the 7b coding sequence. Ultimately, these studies together with the three

dimensional structure of 7b will give more insight into the biological role of the 7b protein.

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